PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PC-2008351	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/SE99/02446	International filing date *(day month year)* 22.12.1999	Priority date *(day month year)* 29.12.1998
International Patent Classification (IPC) or national classification and IPC7 C07K 14/56, C12P 21/02		
Applicant BIONATIVE AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17.05.2000	27.03.2001
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88	Authorized officer Yvonne Siösteen/BS Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE99/02446

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:

pages 1-12,15 , as originally filed

pages _____ , filed with the demand

pages 13-14 , filed with the letter of 24.01.2000

☒ the claims:

pages _____ , as originally filed

pages _____ , as amended (together with any statement) under article 19

pages _____ , filed with the demand

pages 16-17 , filed with the letter of 24.01.2000

☒ the drawings:

pages 1-3 , as originally filed

pages _____ , filed with the demand

pages _____ , filed with the letter of _____

☐ the sequence listing part of the description:

pages _____ , as originally filed

pages _____ , filed with the demand

pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☒ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE99/02446

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9	YES
	Claims		NO
Inventive step (IS)	Claims	1-9	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-9	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The claimed invention relates to a process for production of alpha interferon in human leukocytes induced by virus. The leukocytes are treated with an enhancing agent choosen from xanthine, pyrimidinol, pyrimidinone, yheophylline, theobromine, enprophylline, hypoxanthine, 8-phenyltheophylline, 2-amino-5-bromo-6-methylpyrimidinol, 2-amino-6-methyl-4-pyrimidinol and thymine. The organic solvent is selected from acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinee, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

During the search the following documents were found:

A) EP A1 0048283

B) J.Gen Virol.,Vol 49, 1980, p.91-96.

C) EP A2 0097353

D) US A 3932617

Document A discloses the production of interferon by adding sodium butyrate and N-methyl-2-prrolidinone to lymphoblastoid cells. (see page 4 and example 1).

The use of theophylline for enhancing the production of interferon in tumour cells is known from document B.

It is further known from document C to use theophylline in epithelium cells to enhance the production of interferon.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Box V

In vivo induction of interferon by 2-amino-5-bromo-6-methylpyrimidinol (ABMP) is known from document D.

However, none of the documents disclose the production of interferon in leukocytes. The cited documents are therefore considered to constitute the state of the art. The difficulties in foreseeing which agent is effective in which cells contribute to the opinion that it is not considered obvious to find out that the above mentioned enhancing agents are effective in leukocytes.

Therefore the claims are novel and also considered to involve an inventive step.

NN-dimethylacetamide, NMP, tetramethylene sulfoxide (2.5 mL/L), TMU (1.5 mL/L), 2-pyrrolidinone (1.25 mL/L), 4-metyl-2-pentanone, 1,3-dimetyl-2-imidazolidinone and N-ethyl-2-pyrrolidinone (0.6 mL/L). The incubation is then allowed to proceed overnight. The cells are removed by a centrifugation step and the supernatants are analysed by an ELISA to quantify α-interferon.

The results are shown in Figure 1A.

The above incubation conditions are used for the example shown in Figure 1B, but in this case some of the organic solvents are combined with 50 to 100 μg/mL of 2-ABMP.

Example 2

α-interferon production in Sendai virus induced human leukocytes after incubation with purine and pyrimidine derivatives, in the absence or presence of an organic solvent.

Example 1 is repeated with the same incubation conditions but with addition of one purine and two pyrimidine derivatives either alone or in combination with NMP. The purine used is theophylline (50 μg/ml) and the pyrimidines are 2-ABMP (50 μg/ml) or thymine (140 μg/ml).

The results are shown in Figure 2.

Example 3

α-interferon production in Sendai virus induced human leukocytes after incubation with theophylline and an organic solvent at varying concentrations.

The procedure from example 1 is repeated but with addition of the organic solvent DMSO and the purine theophylline. DMSO is added at 2.5 mL/L or 5 mL/L. The theophylline concentration varies between 5 μg/mL to 100 μg/mL.

The results are shown in Figure 3.

Example 4

α-interferon production in Sendai virus induced human leukocytes incubated in laboratory scale fermentors

An up-scaling is performed where the results obtained in the small scale is used to find suitable conditions for the laboratory fermentors. Different experiments are made to compare the α-interferon production in a fermentor where NMP and theophylline are added with a reference fermentor where no enhancing agent is added. The experiments are performed in laboratory scale fermentors using 2 L medium per vessel. The amount of theophylline is 50 μg/mL, while NMP varies from 1.75 mL/L to 3 mL/L. The incubation conditions are in accordance with Example 1. The results are shown in Figure 4.

Example 5

α-interferon production in human leukocytes incubated with 2-ABMP and DMSO. Effects of virus induction.

Example 1 is repeated with the same incubation conditions but with addition of 2-ABMP in combination with DMSO. The enhancing agents are added both to Sendai virus induced leukocytes and to non induced leukocytes and the effect on the α-interferon production is compared. The 2-ABMP is added at a concentration of 50 μg/ml and the amount of DMSO added is 5 mL/L.

The results are shown in Table 1.

CLAIMS

1. A process for the production of α-interferon comprising the steps:

i) inducing of human leukocytes by means of a virus,

ii) treating the leukocytes with an enhancing agent selected from

a) xanthine, pyrimidinol, pyrimidinone, theophylline, theobromine, enprophylline, hypoxanthine, 8-phenyltheophylline, 2-amino-5-bromo-6-methylpyrimidinol, 2-amino-6-methyl-4-pyrimidinol and thymine;

b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides;

or a combination of the compounds from a) with an organic solvent from b).

2. A process according to claim 1, characterized in that the leukocytes are monocytes.

3. A process according to any one of claims 1 and 2, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

4. A process according to any one of claims 1 - 3, characterized in that the virus is Sendai virus.

5. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is theophylline.

6. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

7. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is thymine.

8. A process according to any one of the preceding claims, characterized in that the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

9. A process according to claim 8, characterized in that the solvent is N-methyl-2-pyrrolidinone.

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/56, C12P 21/02 — A1

(11) International Publication Number: **WO 00/39163**

(43) International Publication Date: 6 July 2000 (06.07.00)

(21) International Application Number: PCT/SE99/02446

(22) International Filing Date: 22 December 1999 (22.12.99)

(30) Priority Data:
9804583-4 29 December 1998 (29.12.98) SE

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(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MODIFICATION OF INTERFERON ALPHA PRODUCTION

(57) Abstract

A process for the production of α-interferon comprising the steps: i) inducing of human leukocytes by means of a virus; ii) treating the leukocytes with an enhancing agent selected from: a) Xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof, such as theophylline, 2-amino-5-bromo-6-methyl-4 pyriminidol or thymine; b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides, such as N-methyl-2-pyrrolidinone, acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, 4-methyl-2-pentanone-N-ethyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide or N,N,-dimethylacetamide; or a combination of the compounds from a) with an organic solvent from b).

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MODIFICATION OF INTERFERON ALPHA PRODUCTION

Field of invention

The present invention is directed to a process for the production of α-interferon in human leukocytes induced by virus. More particularly the invention relates to a process of production of α-interferon in which process the leukocytes are treated with an enhancing agent.

Background of the invention

The Interferons constitutes a family of proteins characterized by their non-specific antiviral and antiproliferative activity, a property that has made them useful as antiviral and anticancer drugs. Interferons are produced and released by animal cells upon exposure to a variety of inducing agents, the most potent of which are viruses. They are classified into three types: α-Interferon, β-Interferon, and γ-Interferon, based on serological and structural relations. The use of interferons as therapeutic drugs dates back to the 1970's. Although all three types of interferons have been subject to evaluation, α-interferon has become most widely used for therapeutic purposes. Among the interferons of human origin, the α-interferons are divided into several *subtypes*, which are either encoded by different gene loci or alleles of those, while there is only one subtype each of human β- and γ-interferon. The function of each subtype is still not clear, and the molecular or cellular targets of their antiviral and antineoplastic activities is thus not fully investigated. However, some *in vitro* studies have shown a larger biological effect when a mixture of different subtypes was used compared to using a single subtype only (Fan, S.X, Skillman, D.R, Liao, M-J, Testa, D. and Meltzer,

M.S. (1993) *AIDS Res. And Human Retrovir.* ***9,*** 1115-1122, Heim, A., Brehm, C., Stille-Siegener, M., Müller, G., Hake, S, Kandolf, R. and Figulla, H-R. **(1995)** *J. Mol. Cell Cardiol.*. ***27,*** 2199-2208).

There are presently three major methods for industry-scale production of α-interferon, all with fundamental differences in the cell system used. In the procaryotic systems, the gene coding for a single subtype, almost exclusively interferon α2, has been transferred to *Escherichia coli*, whereby this protein is expressed by the bacteria and subsequently harvested (Wessmann,C., Hagata,S., Boll,W., et al **(1982)** *Phil. Trans. Royal Soc. London, Series B: Biol. Sci. 299* 7-28). This process for the production of α-interferon is referred to herein as "recombinant interferon". The bacterial cultures can be used for producing α-interferon at high efficiency, leading to an economically advantageous alternative to cells of human origin. However, only one single subtype is produced and the proteins are not modified (e.g. glycosylated) in contrast to interferon α2 that is produced in eukaryotic cells. A clinical drawback encountered with the recombinant α-interferon products is their tendency to induce antibodies against α-interferon in some patients. These neutralising antibodies have in several publications been shown to negatively affect the therapeutic treatment with recombinant α-interferon (Antonelli, G., Simeoni, E., Currenti, M., DePisa, F., Colizzi, V., Pistello, M., and Dianzani, F., **(1997)** *Biother* **10**, 7-14, Öberg, K. and Alm, G. **(1997)** *Biother* **10**, 1-5).

Alternatively α-interferon can be produced in human cells, either from established cell lines which are grown *in vitro*, or from primary cells, e.g., from peripheral leukocytes obtained as by-products from donated blood. In these case a mixture of α-interferon subtypes is

obtained, although different cell sources produce a different subtype pattern. (Goren, T., Fischer, D.G. and Rubinstein M. **(1986)** *J. Interferon* Res. 6, 323-329. Established cell lines are clones derived from human tumors or from cells that have been immortalised, e.g., by the treatment with Epstein-Barr virus. These cells divide and grow indefinitely, in suitable media and under appropriate conditions. In contrast, interferon-producing primary cells such as leukocytes do not divide and have a finite life span. Such cells are consequently in limited supply, and their availability is a limiting factor for large scale production of native leukocyte α-interferon. Means for increasing the yield in the production process are therefore necessary.

Eucaryotic cell systems produce very little or no α-interferon spontaneously. The use of an "*inducer*" is therefore needed to initiate the production of α-interferon by the cells. Consequently, a large number of factors have been reported to initiate the production of α-interferon in various *in vitro* cell systems or *in vivo*. The most common inducers are different viruses, but synthetic organic substances have also been shown to act as inducers for the production of α-interferon. Some examples are leu-enkephalin and naloxone *in vivo* in mice (Gabrilovac, J., Ikic-Sutlic, M., Knezevic, N. and Poljak, L. **(1996)** *Res. Exp. Med.* ***196,*** 137-144), neovir *in vivo* in mice (Mezentseva M., Narovlyansky, A., Kondratieva, T and Ershov, F. **(1997)**. *J.Interferon Res.* ***17, Suppl.2,*** S94. Abstract), dipyridamole *in vivo* in humans (Galabov A.S. and Mastikova M. **(1984)** *Biomed. Pharmacoth.* ***38,*** 412-413), 2-amino-5-bromo-6-methyl-4-pyrimidinol (2-ABMP) and its derivatives *in vivo* in humans(US Patent No 3,932,617, Stringfellow, D.A., Vanderberg, H.C. and Weed, S.D. **(1980)** *J. Interferon Res.* ***1,*** 1-14) and antraquinone derivatives *in vivo* in a variety of species (US patent 4,027,021). Ethanol is

reported to induce β-interferon production in Madin-Darby bovine kidney (MDBK) cells in the absence of other inducers (Chelbi-Alix, M.K. and Chousterman, S. **(1992)** *J. Biol. Chem.* ***267,*** 1741-1745).

There are some examples where the production of α-interferon has been increased by ancillary reagents, termed herein as "*enhancers*", i.e., compound(s) which is/are capable of increasing the production of α-interferon in cells activated by an inducing agent, but does not itself induce production of α -interferon. Characteristic of an enhancer is that it can be added either before the agent that induces α-interferon, or after the induction has taken place. Substances that are reported as enhancers of interferon production in human cells are, e.g., the calmodulin inhibitor trifluoperazine used on fibroblasts (Lin, H-Y. and Thacore, H.R. **(1990)** *J. Interferon Res.* ***10,*** 375-378), dexamethasone used as a stimulator on a cell line of lymphoblastoid origin (US patent 4,266,024) and sodium butyrate, also used as a stimulant in a lymphoblastoid cell line (EP 0097 353, EP 0000520). Furthermore, it has been reported that the purine derivative theophylline acts as an enhancer and increases the yield of α-interferon from mouse Lpa cells induced by poly I:C (Zahorska, R., Korbecki, M., and Barciszewski, J. **(1995)** *Arch. Immunol. Ther. Exp.* ***43,*** 43-46), and the use of some synthetic organic compounds, preferably tetramethylurea (TMU) (European Patent No 0 097 353), or dimethylsulfoxide (DMSO) (US Patent No 4,266,024) has been shown to increase the α-interferon yield from a lymphoblastoid cell line treated with an inducer.

In the work leading to the present invention some of the substances which has been reported as inducers or enhancers when used in vivo or on fibroblasts and on a cell line of lymphoblastoid origin has been tested on

human leukocytes, with the aim to increase the α-interferon production, both before or after induction with Sendai virus, but without any positive result on the yield of α-interferon. In some experiments even a decrease in α-interferon production from human leukocytes was observed when adding compounds known as enhancers in cell lines. For instance, pre-treatment with sodium butyrate has been described as a stimulant that leads to an increased yield of α-interferon in a lymphoblastoid cell line (European Patent No 0 097 353; European Patent No 0 000 520). In the present work it was found that pre-treatment of primary leukocytes with sodium butyrate according to a similar procedure led to a *decrease* in the yield of α-interferons, which points at fundamental differences between cell lines and primary cells as production systems for α-interferon proteins.

It is accordingly an object of the present invention to provide an improved process for production of α-interferon in human leukocytes induced by virus.

Summary of the invention

The object of the invention is obtained by the process for the production of α-interferon as claimed in the claims.

According to the invention there is provided a process for the production of α-interferon comprising the steps:

i) inducing of human leukocytes by means of a virus,

ii) treating the leukocytes with an enhancing agent selected from

a) Xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof;

b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides,

alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides; or a combination of the compounds from a) with an organic solvent from b).

According to the present invention, it was surprisingly found that the addition of the compounds and/or an organic solvent as indicated above, increases the amount of virus induced α-interferon in leukocytes. Considering the different species in the case of the mouse Lpa cells and the different cell source in the case of the lymphoblastoid cell line, and considering the lack of consistency between the different cell systems in the experiments described above, it was surprisingly found that theophylline as well as a solvent such as dimethylsulfoxide enhanced the production of α-interferon in virus induced primary leukocytes. Also, the pyrimidine derivative 2-amino-5-bromo-6-methyl-4-pyrimidinol (2-ABMP), was found to act as an enhancing agent of the α-interferon production according to the present invention using virus induced primary leukocytes. Since 2-ABMP did not induce α-interferon in the absence of a viral inducer using the experimental system described in this invention, the mechanism for enhancement is obviously different for the *in vivo* α-interferon induction described in the literature, as compared to the α-interferon production enhancing effect of this substance observed in the present invention. This further strengthens the evidence that different mechanisms are in effect in different human cell systems, and indicates the unpredictiveness in working with additives to cellular systems or organisms.

As mentioned above several agents have been used as inducers and enhancers in both in vivo systems and in different cell culture systems. Since the data in the literature in many cases deviated from the findings in the present invention e.g. the inability for 2-ABMP to

act as an interferon inducer in the cell system according to the invention, several unique properties of the cellular system in this invention should be pointed out. Primary cells from peripheral blood represent a resting cell population in contrast to all the other cellular systems described for interferon production. The cells are not activated or immortalised by virus, as is the case for Burkitt's lymphoma cells or so called lyphoblastoid cells. Nor are they tumor cell lines with their inherent genomic instability and dysregulated gene expression. It has been shown that within a mixture of leukocytes derived from human blood the monocytes are the main producers of α-interferon after Sendai virus treatment (Sandberg, K., Matsson, P. And Alm, G. **(1990)** *J. Immunology* **145,** 1015-1020). Thus, the cells used in this invention differ from the established cell lines used for α-interferon production with respect to growth state, neoplastic potential, presence of viral genome and cell type. The effects of the tested factors are therefore a priori not expected to be the same as for other cell types, with differences in differentiation, metabolic state or interference from viral genomes.

Detailed description of the invention

According to the present invention it was found that addition of enhancing agents in the form of xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof and/or various organic solvents significantly increases the interferon production in human leukocytes induced by virus. The enhancing agent can increase the interferon yield either alone or in a combination of an organic solvent and the mentioned compounds. At certain conditions the effect of combining these enhancing agents is synergistic, showing a larger effect than the one obtained using the substances separately.

The xanthine derivatives that can be used according to the invention are xanthine with aliphatic and/or aromatic substituens, such as theophylline, theobromine, enprophylline, hypoxanthine and 8-phenyltheophylline. Theophylline is a preferred enhancing agent. Pyrimidinol derivatives that can be used are for example 2-amino-5-bromo-6-methyl-pyrimidinol and 2-amino-6-methyl-4-pyrimidinol with 2-amino-5-bromo-6-methyl-pyrimidinol as a preferred embodiment. A preferred pyrimidinone derivative is thymine.

The organic solvents that can be used according to the invention are non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides. As preferred solvents can be mentioned acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone (NMP), 2-pyrrolidinone, tetramethylene sulfoxide, N,N-dimethylacetamide, 2-pyrrolidinone. The most preferred compounds presented under item a) above with respect to interferon production is theophylline and 2-ABMP. The most preferred solvent is NMP.

The virus used can be any virus, but the preferred virus is Sendai virus, the most potent inducer of interferon-α for purified human leukocytes.

Leukocyte purification, incubation of the cells and induction by virus are mainly performed according to the original method of Cantell et al. (Cantell, K., Hirvonen, S., Kauppinen, H-L. and Myllylä, G. (1981) Methods in Enzymology 78, 29-38).

The human leukocytes used according to the invention are prepared from buffy coats by an initial centrifugation to

fractionate the components, followed by sequential removal of the plasma layer and of the leukocyte fraction by suction. Residual red blood cells contaminating the leukocyte fraction are lysed twice with 2 - 4 volumes of cold 0.83 % ammonium chloride and each lysis step is followed by centrifugation to collect the cells. The final leukocyte fraction is resuspended in a basal medium, e.g. EMEM (Eagle's minimum essential medium) supplemented with polyethylene glycol- (PEG)-precipitated human plasma. PEG-precipitated plasma is prepared by adding PEG 6000 to a final concentration of 6 % (w/w) to human plasma. After precipitation for 3-5 days in a cold room the supernatant is removed and stored frozen until used. The temperature of the incubation medium is set to 36 -37°C and continuous stirring is used. Human PEG-precipitated plasma is added to a concentration of 1 - 5 % (v/v), preferably 4 % (v/v). A low amount of α-interferon (normally 100 IU/ml) is added to the incubation medium as a priming step, but other concentrations, up to several thousands of units per ml can be used. Human leukocytes are then added to a concentration of about 3 - 15 million cells per mL, preferably 7 - 11 million cells per mL. The similar size of the enhancing effect observed using the different cell concentrations makes it probable that the effect can be seen also at cell concentrations outside this range. The priming step is normally allowed to proceed for 1 - 5 hours, preferably 1.5 - 2 hours.

Induction of interferon is performed by addition of Sendai virus, generally 30 mL virus from allantoic fluid per litre incubation medium but both smaller and larger amounts showed good inducing capacity and the most

preferred range is 500-2000 hemagglutinating virus particles per cell. A decrease of the incubation temperature to 29°C - 32 °C is performed (Morser, J. And Shuttleworth, J. **(1981)** *J. Gen. Virology* ***56,*** 163-174) after another 1 to 4 hours, preferable after 1 to 2 hours.

The optimal timing for adding the different enhancing agents varies, but in general an increase in α-interferon production is seen when the substances are added either at the same time as the cells or up to several hours after the induction, preferentially close to the time of the temperature decrease.

The concentration range where the additives are effective in increasing the interferon production varies between the different compounds but for the least toxic solvents a positive effect can be seen in the range of 1 mM - 0.3 M, preferably in the range of 3 mM - 50 mM and most preferably within 5 mM - 20mM. For the more toxic solvents the suitable concentration lies within the more limited ranges. For the theophylline, 2-amino-5-bromo-6-methyl-4-pyrimidinol and thymine the effective range is 5 μM - 0.5 mM, preferably 20 μM - 0.15 mM. After these additions, the incubation is allowed to proceed overnight with continuous stirring. A centrifugation step is performed to remove the cells and the supernatants are analysed by an ELISA to quantify α-interferon.

The invention will now be illustrated with the following non-limiting examples and with reference to the figures:

Figure legends

Figure 1A:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different organic solvents to the incubation medium. The amount of α-interferon produced is calculated as percent of a reference with no addition of organic solvents (=100 %).

Figure 1B:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different organic solvents in combination with 2-ABMP to the incubation medium. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agent (=100 %).

Figure 2:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different purine and pyrimidine derivatives at different NMP concentrations. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Figure 3:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different amounts of theophylline at different DMSO concentrations. The amount

of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Figure 4:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of NMP and theophylline to the incubation medium in laboratory scale fermentors. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Table 1

Effect on α-interferon production of addition of enhancing agents, 2-ABMP and DMSO, both to Sendai virus induced human leukocytes and to non induced human leukocytes.

Example 1

α-interferon production by Sendai virus induced human leukocytes after incubation with various organic solvents.

Human leukocytes prepared from buffy coats and used in concentrations between 8 and 11 million cells per mL are incubated in a basal media (either EMEM or a modified EMEM) supplemented with 1.5 g/L of tricine and 4 % (v/v) PEG precipitated plasma. The experiments are performed in a volume of 40 mL medium in 100 mL glass bottles under continuous stirring. The cells are primed with 100 IU/mL α-interferon for 1.5 hours at 37°C before addition of 30 mL/L Sendai virus. After 1.5 hours the incubation temperature is decreased to 30°C and the organic solvents are added to the incubation medium. The solvent used are acetone, DMSO (final concentration: 5 mL/L), 2-butanone,

NN-dimethylacetamide, NMP, tetramethylene sulfoxide (2.5 mL/L), TMU (1.5 mL/L), 2-pyrrolidinone (1.25 mL/L), 4-metyl-2-pentanone, 1,3-dimetyl-2-imidazolidinone and N-ethyl-2-pyrrolidinone (0.6 mL/L). The incubation is then allowed to proceed overnight. The cells are removed by a centrifugation step and the supernatants are analysed by an ELISA to quantify a-interferon.

The results are shown in Figure 1A.

The above incubation conditions are used for the example shown in Figure 1B, but in this case some of the organic solvents are combined with 50 to 100 mg/mL of 2-ABMP.

Example 2

α-interferon production in Sendai virus induced human leukocytes after incubation with purine and pyrimidine derivatives, in the absence or presence of an organic solvent.

Example 1 is repeated with the same incubation conditions but with addition of one purine and two pyrimidine derivatives either alone or in combination with NMP. The purine used is theophylline (50 mg/ml) and the pyrimidines are 2-ABMP (50 mg/ml) or thymine (140 mg/ml).

The results are shown in Figure 2.

Example 3

α-interferon production in Sendai virus induced human leukocytes after incubation with theophylline and an organic solvent at varying concentrations.

The procedure from example 1 is repeated but with addition of the organic solvent DMSO and the purine theophylline. DMSO is added at 2.5 mL/L or 5 mL/L. The theophylline concentration varies between 5 mg/mL to 100 mg/mL.

The results are shown in Figure 3.

Example 4

α-interferon production in Sendai virus induced human leukocytes incubated in laboratory scale fermentors

An up-scaling is performed where the results obtained in the small scale is used to find suitable conditions for the laboratory fermentors. Different experiments are made to compare the α-interferon production in a fermentor where NMP and theophylline are added with a reference fermentor where no enhancing agent is added. The experiments are performed in laboratory scale fermentors using 2 L medium per vessel. The amount of theophylline is 50 mg/mL, while NMP varies from 1.75 mL/L to 3 mL/L. The incubation conditions are in accordance with Example 1. The results are shown in Figure 4.

Example 5

α-interferon production in human leukocytes incubated with 2-ABMP and DMSO. Effects of virus induction.

Example 1 is repeated with the same incubation conditions but with addition of 2-ABMP in combination with DMSO. The enhancing agents are added both to Sendai virus induced leukocytes and to non induced leukocytes and the effect on the α-interferon production is compared. The 2-ABMP is added at a concentration of 50 mg/ml and the amount of DMSO added is 5 mL/L.

The results are shown in Table 1.

Table 1

Viral induction	Enhancing agent	Yield of α-interferon
Sendai virus (30 mL/L)	None	100 %
Sendai virus (30 mL/L)	2-ABMP (50 μg/mL) and DMSO (5 mL/L)	146 %
None	2-ABMP (50 μg/mL) and DMSO (5 mL/L)	< 1 %

Conclusion

In the present invention it has been shown that addition of different organic solvents as well as different purines and pyrimidines reproducibly increases the production of α-interferon in virus induced human leukocytes. The largest increase is obtained when the purine or pyrimidine derivatives are combined with an organic solvent.

Claims

1. A process for the production of α-interferon comprising the steps:

i) inducing of human leukocytes by means of a virus,

ii) treating the leukocytes with an enhancing agent selected from

a) Xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof;

b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides; or a combination of the compounds from a) with an organic solvent from b).

2. A process according to claim 1, characterized in that the leukocytes are monocytes.

3. A process according to claim 1 or 2, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

4. A process according to claim 1 - 3, characterized in that the virus is Sendai virus.

5. A process according to claim 1 - 4, characterized in that the xanthine derivative is xanthine with aliphatic and/or aromatic substituents.

6. A process according to claim 5, characterized in that the xanthine derivative is theophylline.

7. A process according to claim 1 - 4, characterized in that the pyrimidinol derivative is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

8. A process according to claim 1 - 4, characterized in that the pyrimidinone derivative is thymine.

9. A process according to claim 1 - 4, characterized in that, the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide, N,N-dimethylacetamide, 2-pyrrolidinone.

10. A process according to claim 1 - 4, characterized in that the solvent is N-methyl-2-pyrrolidinone.

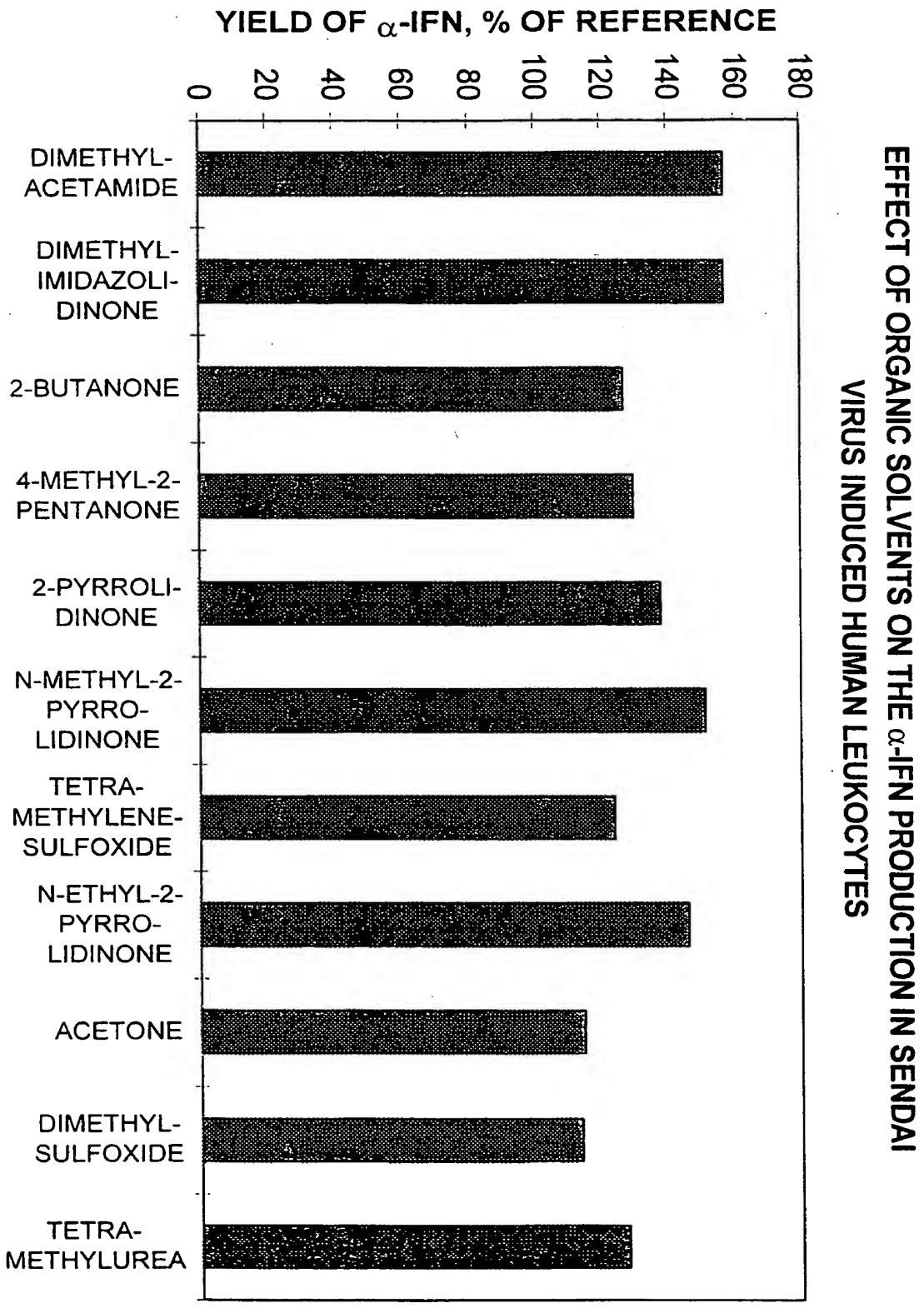
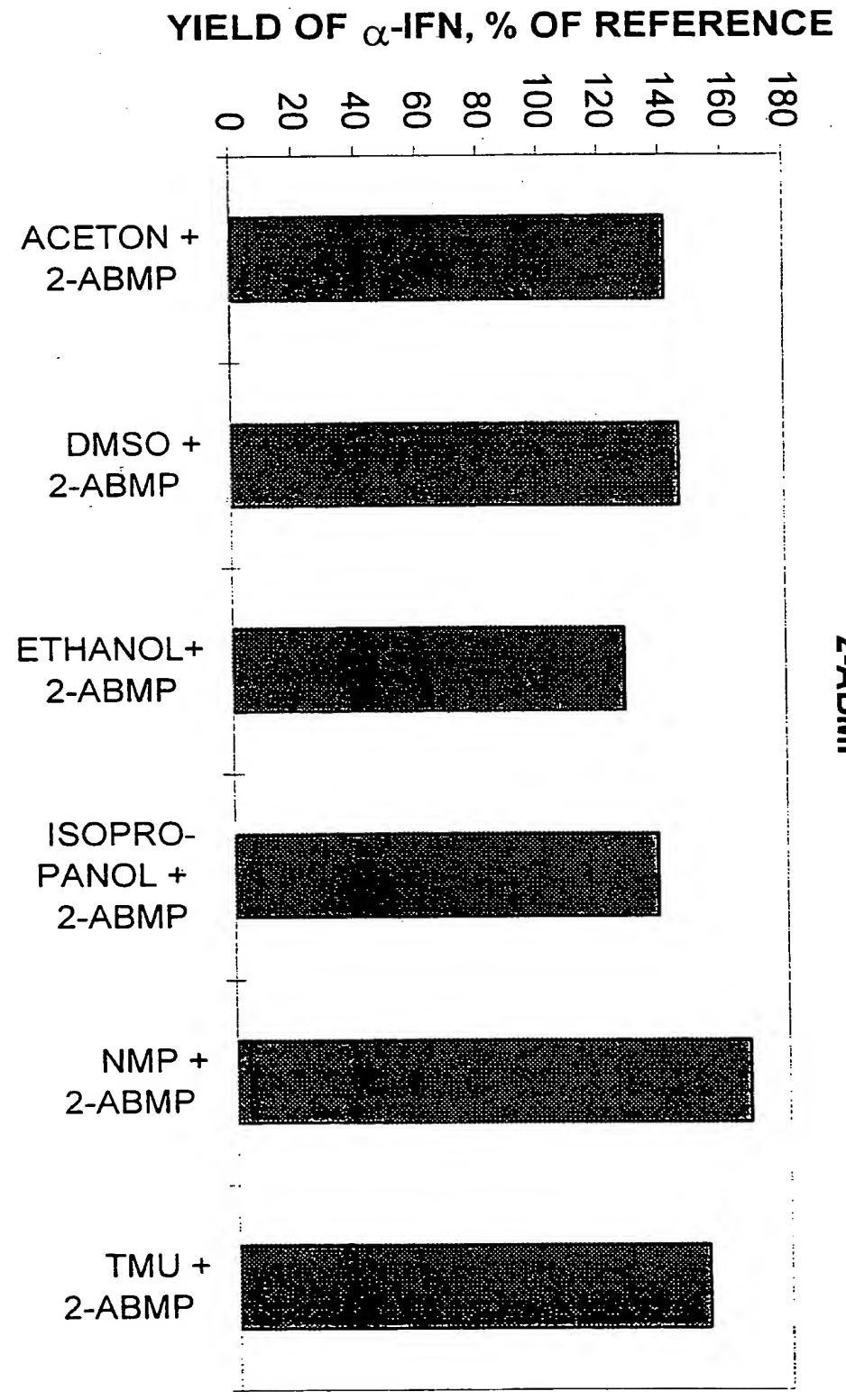

YIELD OF α-IFN, % OF REFERENCE
0
20
40
60
80
100
120
140
160
180
DIMETHYL-ACETAMIDE
DIMETHYL-IMIDAZOLI-DINONE
2-BUTANONE
4-METHYL-2-PENTANONE
2-PYRROLI-DINONE
N-METHYL-2-PYRRO-LIDINONE
TETRA-METHYLENE-SULFOXIDE
N-ETHYL-2-PYRRO-LIDINONE
ACETONE
DIMETHYL-SULFOXIDE
TETRA-METHYLUREA
EFFECT OF ORGANIC SOLVENTS ON THE α-IFN PRODUCTION IN SENDAI VIRUS INDUCED HUMAN LEUKOCYTES

FIGURE 1A



EFFECT ON α-IFN PRODUCTION IN SENDAI VIRUS INDUCED HUMAN LEUKOCYTES BY DIFFERENT ORGANIC SOLVENTS COMBINED WITH 2-ABMP

FIGURE 1B

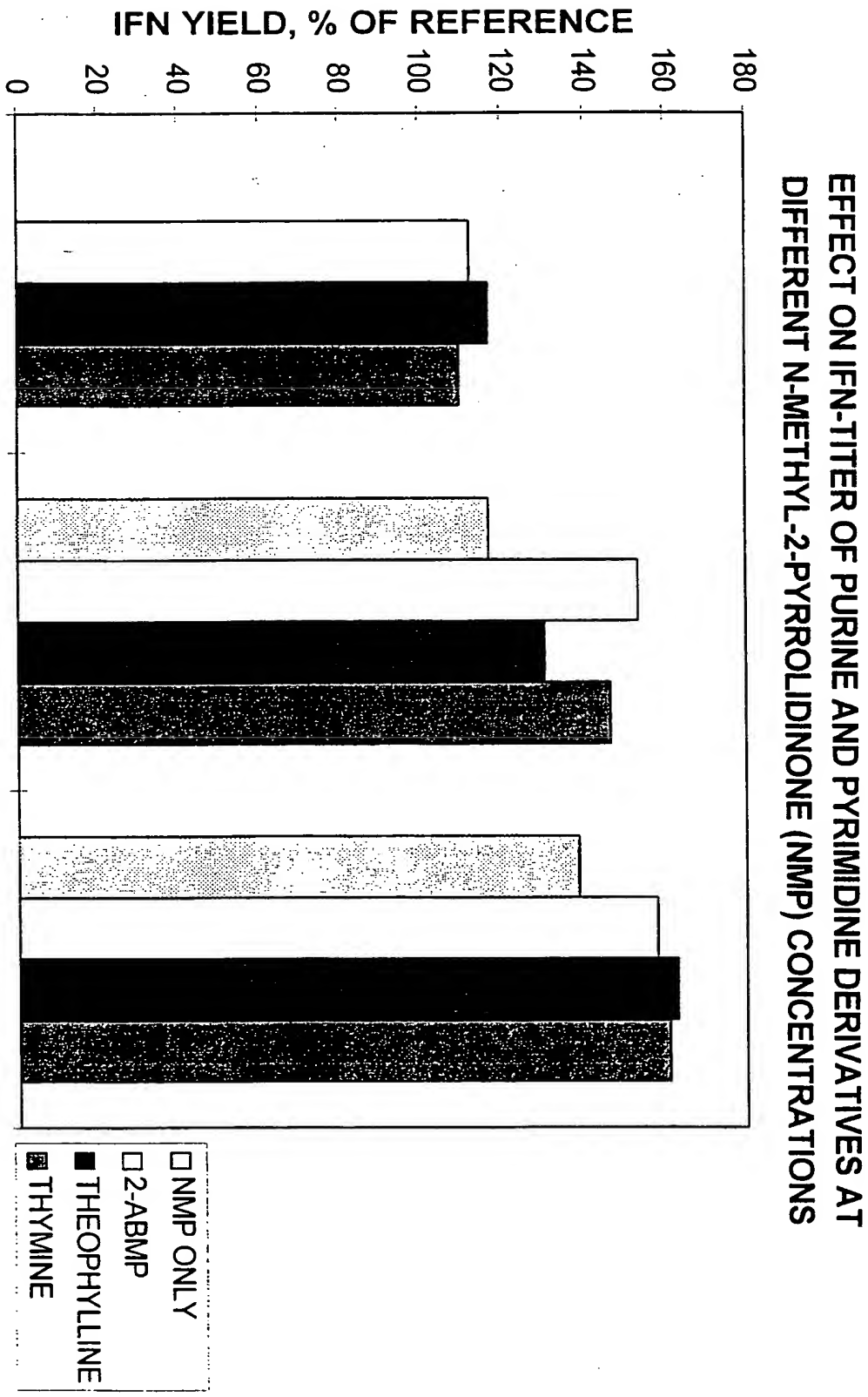

EFFECT ON IFN-TITER OF PURINE AND PYRIMIDINE DERIVATIVES AT DIFFERENT N-METHYL-2-PYRROLIDINONE (NMP) CONCENTRATIONS
IFN YIELD, % OF REFERENCE
0
20
40
60
80
100
120
140
160
180
NMP ADDED (mL/L)
0
0.25
0.62
NMP ONLY
2-ABMP
THEOPHYLLINE
THYMINE

FIGURE 2

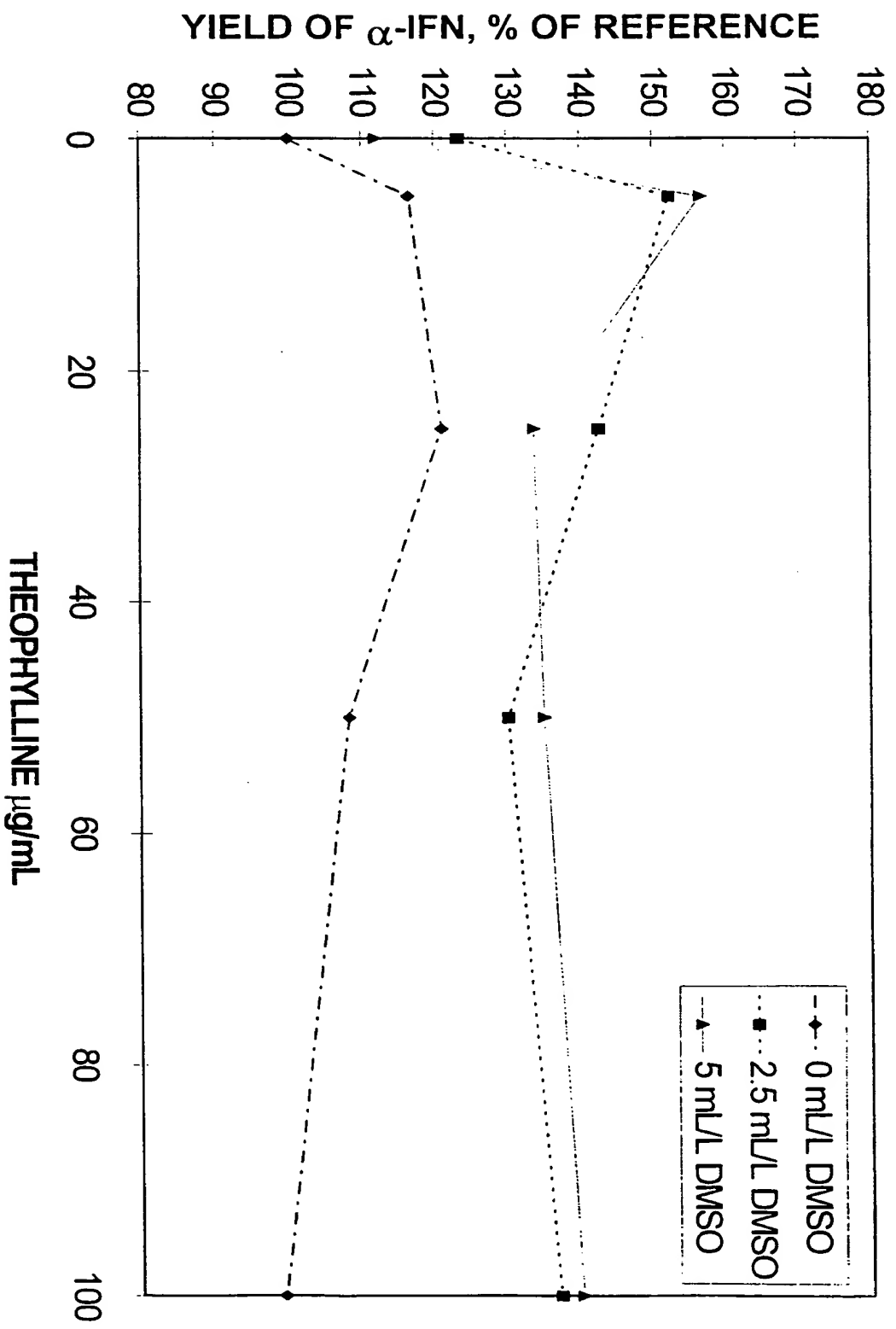

EFFECT OF INCREASING AMOUNTS OF THEOPHYLLINE AT DIFFERENT DMSO CONCENTRATIONS
YIELD OF α-IFN, % OF REFERENCE
80
90
100
110
120
130
140
150
160
170
180
0
20
40
60
80
100
THEOPHYLLINE µg/mL
0 mL/L DMSO
2.5 mL/L DMSO
5 mL/L DMSO

FIGURE 3

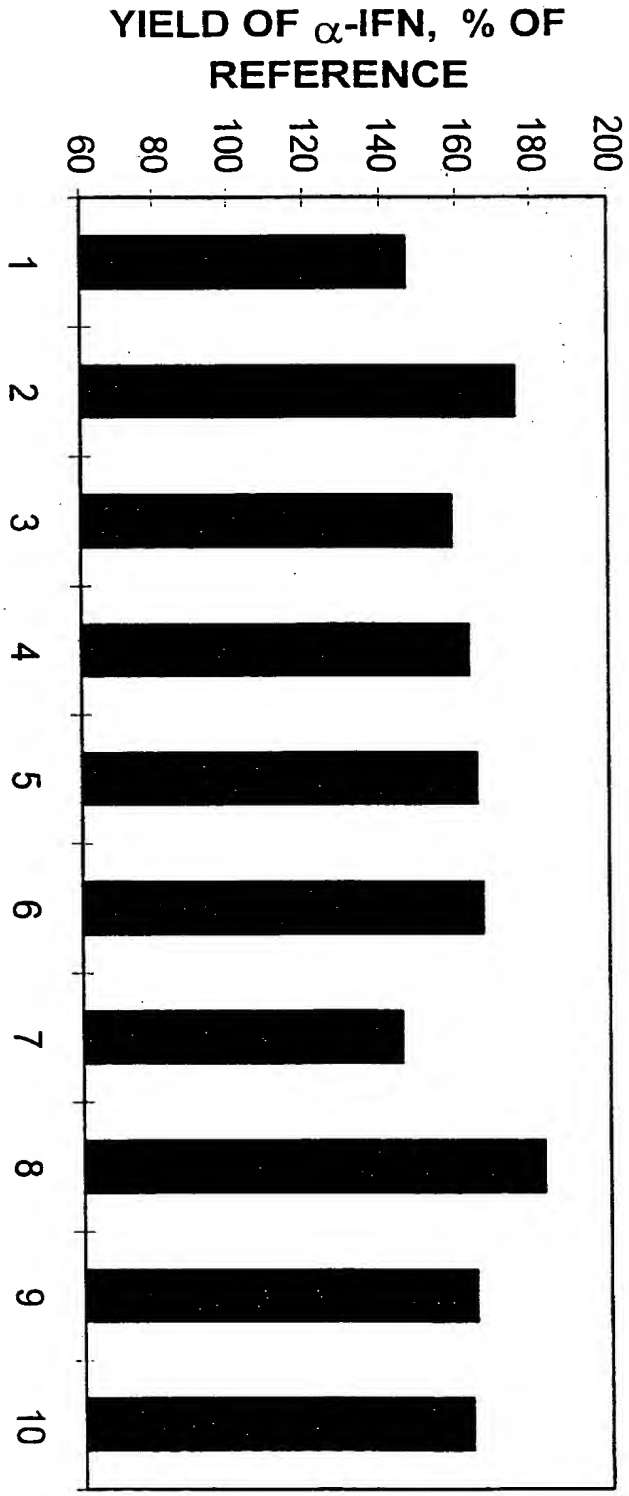

EFFECT OF NMP AND THEOPHYLLINE ON α-INTERFERON PRODUCTION IN SENDAI VIRUS INDUCED LEUKOCYTES INCUBATED IN LABORATORY FERMENTORS
YIELD OF α-IFN, % OF REFERENCE
60
80
100
120
140
160
180
200
1
2
3
4
5
6
7
8
9
10
EXPERIMENT NUMBER

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/02446

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/56, C12P 21/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0048283 A1 (TAGUCHI, FUMIAKI), 31 March 1982 (31.03.82), see page 7, line 25 - page 8, line 21 and claims --	1-6
A	J. gen. Virol., Volume 49, 1980, E. Slattery et al, "Mouse Interferons: Production by Ehrlich Ascites Tumor Cells Infected with Newcastle Disease Virus and its Enahncement by Theophylline", page 91 - page 96, see esp. pages 94-95 --	1-6
A	EP 0097353 A2 (THE WELLCOME FOUNDATION LIMITED), 4 January 1984 (04.01.84), see page 3, line 31 - page 5, line 7 --	1-4,9,10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2000	27 -04- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/02446

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3932617 A (FRANCIS RICHARD NICHOL, JR. ET AL), 13 January 1976 (13.01.76), see claim 4 -- --------	1,7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE99/02446

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1 (partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next sheet

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Unsearchable

The present claim 1 relates to an extremely large number of possible methods. Support within the meaning of Article 6 and disclosure within the meaning of Article 5 is to be found, however, for only a very small proportion of the methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claim which appear to be supported and disclosed, namely those parts relating to methods for production of alpha interferon in human leukocytes comprising the use of an enhancing agent selected from xanthine, pyrimidinol, pyrimidinone, theophylline, theobromine, enprophylline, hypoxanthine, 8-phenyltheophylline, 2-amino-5-bromo-6-methylpyrimidinol, 2-amino-6-methyl-4-pyrimidinol and thymine, and /or an organic solvent selected from acetone, 2-butanone, 1, 3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N, N-dimethylacetamide.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 99/02446

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0048283 A1	31/03/82	AU 538563 B	16/08/84
		AU 6924881 A	09/10/81
		CH 646875 A	28/12/84
		GB 2083359 A,B	24/03/82
		JP 56135420 A	22/10/81
		NL 8120080 T	01/02/82
		SE 8106944 A	23/11/81
		WO 8102674 A	01/10/81
EP 0097353 A2	04/01/84	SE 0097353 T3	
		AT 55620 T	15/09/90
		AU 572783 B	19/05/88
		AU 1593583 A	05/01/84
		CA 1211397 A	16/09/86
		CS 251766 B	13/08/87
		CS 8304518 A	18/12/86
		DK 283683 A	22/12/83
		ES 523424 A	16/04/85
		FI 832252 A	22/12/83
		GB 2122207 A,B	11/01/84
		IL 69027 A	31/08/88
		IT 1172270 B	18/06/87
		IT 8348536 D	00/00/00
		JP 6036751 B	18/05/94
		JP 59006896 A	13/01/84
		US 4780413 A	25/10/88
US 3932617 A	13/01/76	BE 829418 A	24/11/75
		DE 2522047 A	04/12/75
		FR 2271824 A,B	19/12/75
		GB 1475221 A	01/06/77
		NL 7505942 A	26/11/75
		ZA 7502762 A	28/04/76